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In re

Patent Application of

David L. Epstein, et al.

Application No. 10/580,477

Confirmation No. 4348

Filed: January 25, 2007

Examiner: Zohreh A. Fay

"METHOD OF TREATING OR PREVENTING GLAUCOMA"

DECLARATION UNDER 37 CFR 1.131

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
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Declarant Vasanth Rao hereby declares and states the following:

1. I am a co-inventor of the subject matter of U.S. Patent Application Serial No. 10/580,477, which was filed on January 25, 2007 ("the Application") and claims priority to PCT Application No. PCT/US04/39657, which claims priority to U.S. Provisional Patent Application Serial No. 60/524,912 filed on November 26, 2003 ("the Provisional"). I make this declaration in support of the Application.

2. I understand that the United States Patent & Trademark Office has rejected claims 9, 11-16, 21, 23-29 and 31-35 of the Application as being unpatentable over U.S. Patent Publication No. 2004/0248972 to Lockhart et al. ("Lockhart").

3. I understand that Lockhart was filed on May 17, 2004 and claims priority to U.S. Provisional Patent Application Serial No. 60/471,425, which was filed on May 16, 2003.

4. Prior to May 16, 2003, we conceived of the idea that statins could be used to treat or inhibit the progression of glaucoma and to control normal or elevated intraocular pressure.

5. Prior to May 16, 2003, it was known that elevated intraocular pressure commonly associated with glaucomatous conditions may be a consequence of impairments in trabecular meshwork (TM) function, i.e., the impairment of drainage of aqueous humor from the eye. It was also known that changes in cell shape, i.e., rounding up of cells, and decreases in actin stress fibers and myosin light chain phosphorylation in TM cells correlate well with increased cell relaxation and increased outflow facility. Furthermore, it was also known that compounds that caused changes in cell shape and decreases in actin stress fibers and myosin light chain phosphorylation in TM cell could be used as a treatment for glaucoma. *See e.g., Investigative Ophthalmology & Visual Science*, 42:1029-1037 published in April 2001, attached hereto as Exhibit A.

6. Prior to May 16, 2003, we determined that statins affect cell shape, i.e., cause rounding up of cells, decrease actin polymerization and relaxation of cells depolymerize actin stress fibers and decrease myosin light chain phosphorylation in different cell types including TM cells as described in an email sent by inventor Vasanth Rao prior to May 16, 2003, attached hereto as Exhibit B. Attached hereto as Exhibit C are lab notebook pages dated prior to May 16, 2003 showing control and 30 μM lovastatin-treated TM cells after 24 hours.

7. I, Vasanth Rao, hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 7/22/11

Vasanth Rao

EXHIBIT A

Modulation of Aqueous Humor Outflow Facility by the Rho Kinase–Specific Inhibitor Y-27632

P. Vasantha Rao,[1,2] *Pei-Feng Deng,*[1] *Janardan Kumar,*[1] *and David L. Epstein*[1]

Purpose. The goal of this study was to investigate the role of Rho kinase in the modulation of aqueous humor outflow facility. Rho kinase, a critical downstream effector of Rho GTPase is recognized to control the formation of actin stress fibers, focal adhesions, and cellular contraction.

Methods. Expression of Rho GTPase, Rho kinase, and other downstream targets of Rho GTPase were determined in human trabecular meshwork (HTM) and Schlemm's canal (SC) primary cell cultures by Western blot analysis. The Rho kinase–specific inhibitor (Y-27632)-induced changes in actin stress fibers, focal adhesions, and protein phosphotyrosine status were evaluated by staining with rhodamine-phalloidin, anti-paxillin, and anti-phosphotyrosine antibodies, respectively. Myosin light-chain phosphorylation was determined by Western blot analysis. Y-27632-induced changes in SC cell monolayer permeability were quantitated using a colorimetric assay to evaluate horseradish peroxidase diffusion through SC cell monolayers grown in transwell chambers. Aqueous humor outflow facility was measured using enucleated porcine eyes and a constant-pressure perfusion system.

Results. Treatment of HTM and SC cells with Y-27632 (10 μM) led to significant but reversible changes in cell shape and decreases in actin stress fibers, focal adhesions, and protein phosphotyrosine staining. SC cell monolayer permeability increased (by 80%) in response to Y-27632 (10 μM) treatment, whereas myosin light-chain phosphorylation was decreased in both HTM and SC cells. Aqueous humor outflow facility increased (40%–80%) in enucleated porcine eyes perfused with Y-27632 (10–100 μM), and this effect was associated with widening of the extracellular spaces, particularly the optically empty area of the juxtacanalicular tissue (JCT). The integrity of inner wall of aqueous plexi, however, was observed to be intact.

Conclusions. Based on the Rho kinase inhibitor-induced changes in myosin light-chain phosphorylation and actomyosin organization, it is reasonable to conclude that cellular relaxation and loss of cell–substratum adhesions in HTM and SC cells could result in either increased paracellular fluid flow across Schlemm's canal or altered flow pathway through the JCT, thereby lowering resistanee to outflow. This study also suggests Rho kinase as a potential therapeutic target for the development of drugs to modulate intraocular pressure in glaucoma patients. (*Invest Ophthalmol Vis Sci.* 2001;42: 1029–1037)

Understanding potential regulatory mechanisms controlling aqueous humor outflow facility has important implications for unraveling the etiology of glaucoma and developing better therapy. The elevated intraocular pressure that is commonly associated with glaucomatous conditions is believed to arise as a consequence of impairment(s) in trabecular meshwork function.[1–3] Interestingly, actomyosin and microtubule network interacting agents such as cytochalasin, latrunculin, H-7, ethacrynic acid, BDM, M-9, and vinblastine have all been shown to influence aqueous humor outflow facility.[4–15] Based on these observations, various hypotheses have been advanced to explain the effects of cytoskeletal agents on aqueous humor outflow.[9,13] Furthermore, there is a general consensus that cytoskeletal agents that do not exert toxic effects on ocular tissues might possess therapeutic potential in the treatment of glaucoma.[4]

Cellular contraction and relaxation as well as cell–substratum and cell–cell adhesive forces of human trabecular meshwork (HTM) and SC cells are considered to be important factors in the maintenance of normal aqueous humor outflow facility.[4–13] However, very few studies have attempted mechanistically to identify specific cytoskeletal targets for this. Such studies are important not only to understand the physiological mechanism(s) involved but are also critical to the identification of potential target proteins for rational glaucoma drug design.

We hypothesized that the Rho GTPase-mediated signaling pathway(s) plays an important role in the regulation of trabecular meshwork function and in the maintenance of aqueous humor outflow. The small GTP-binding proteins of the Rho GTPase subfamily are known to be critical regulators of cellular contraction and relaxation, and cell–cell and cell–substratum adhesive interactions, all of which are in turn closely linked to cytoskeletal organization.[15–17]

Several studies have demonstrated that activation of Rho GTPase can promote actin stress fiber assembly, focal adhesion formation, and cellular contraction in fibroblasts.[15,16,18] Rho GTPase regulates all these cytoskeletal events via activation of its downstream targets, Rho kinase and LIM kinase, and the phosphorylation of myosin light-chain and ERM proteins.[16–22] Myosin light-chain phosphorylation plays a pivotal role in the actin–myosin interactions modulating formation of stress fibers, focal adhesions, and cellular contraction.[15,16,18] Rho kinase is implicated in Rho-mediated inhibition of smooth muscle myosin phosphatase activity, on the basis of its ability to phosphorylate and inhibit activity of purified smooth muscle myosin phosphatase in vitro.[22] Rho kinase also regulates cofilin phosphorylation through LIM kinase,[17] thereby abolishing the ability of cofilin to act as an actin-depolymerizing agent. Based on the ability of Rho kinase to modulate the properties and/or function of these cytoskeletal target proteins, Rho GTPase, which directly activates Rho kinase, is thought to play an important role in actomyosin-based contractility.[15–18] Finally, cellular contractility is recognized to underlie the assembly of actin stress fibers and focal adhesions formation.[16,18] Because TM tissues possess smooth muscle cell-like properties and express α-smooth muscle actin by some TM cells, their contractile and relaxation properties are considered to be important in the regulation of outflow.[14,23]

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In our previous study, thrombin and LPA agonists known to stimulate Rho GTPase function were found to stimulate/increase formation of actin stress fibers and focal adhesions as well as increase myosin light-chain phosphorylation in HTM and SC cells maintained as primary cultures and to decrease aqueous humor outflow facility in porcine eyes (Kumar J, Rao PV, Epstein DL, unpublished data). These preliminary studies suggested that Rho GTPase function might be a determinant of cellular contraction in HTM and SC cells, and also that activation of Rho GTPase most likely leads to decreased outflow facility. To evaluate this hypothesis, we studied the effects of Y-27632 ((+)-R-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide), a potent, highly specific, cell-permeable inhibitor of Rho kinase[21,24,25] on actomyosin cytoskeletal organization in HTM and SC cells and evaluated its effects on aqueous humor outflow facility in cadaver porcine eyes. Compound Y-27632 has been found to inhibit both Rho kinase 1 (ROCK I) and Rho kinase 2 (ROCK II) with a K_i of 0.14 to 1.0 μM.[25] Y-27632 has also been shown to reduce systemic hypertension in animal studies, without any toxic side effects.[21]

Materials and Methods

Materials

Wellfide Corporation, Japan, kindly provided the Rho kinase–specific inhibitor Y-27632. Polyclonal antibody directed against myosin light-chain was a gift from Viswanathan Natarajan from Johns Hopkins University (Baltimore, MD). Horseradish peroxidase (HRP), rhodamine-phalloidin, and myosin light-chain monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies used to detect RhoA GTPase and cofilin was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against paxillin and phosphotyrosine and anti-Rho kinase polyclonal antibody were procured from Transduction Laboratories (Lexington, KY). Collagen-coated transwells were from Becton-Dickinson (Bedford, MA). Cell culture media and fetal bovine serum were obtained from Gibco-BRL (Gaithersburg, MD). ECL detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ), and all other chemicals were of analytical grade.

Cell Cultures

HTM and Schlemm's canal cells from cadaver human eyes (obtained from the National Disease Research Interchange, Philadelphia, PA) were isolated as described by Stamer et al.[26] Cells were cultured at 37°C under 5% CO_2, in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS) and penicillin (100 Units/ml)-streptomycin (100 μg/ml). All experiments were conducted using confluent cell cultures. The SC and HTM cells used in this study were isolated from donor eyes of subjects ≤ 30 years of age. Cells were used at passages between 3 to 5.

Cytoskeletal Staining

Both SC and HTM cells were grown to confluence on gelatin (2%)-coated, glass coverslips. Cells were washed twice with serum-free media before treatment with Y27632 (10 μM for 1 hour). Cells were maintained in 10% FBS wherever indicated. Changes in cell shape were recorded with a Zeiss IM 35 phase contrast microscope. After treatment with inhibitor, cells were fixed with 3.7% formaldehyde in cytoskeletal buffer (10 mM MES (2-*N*-morpholino-(ethanesulfonic ether) *N,N,N,N*-tetra acetic acid), 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM glucose, pH 6.1) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) at room temperature. Actin was stained with rhodamine-phalloidin, whereas focal adhesions and phosphotyrosine were stained with primary antibodies raised against paxillin and phosphotyrosine, respectively, followed by use of TRITC-conjugated secondary antibodies. Micrographs were recorded using a Zeiss Axioplan-II fluorescence microscope.

Cell Viability

To evaluate the effects of Y-27632 on viability of HTM and SC cells, cells were grown to confluence on gelatin-coated, glass coverslips, and after treatment with 10 μM drug for 1 hour, cells were rinsed twice with PBS and treated with fluorescein diacetate and propidium iodide for 10 minutes as described by Erickson-Lamy et al.[6] Viable cells and dead/damaged cells, which stain green and red respectively, were checked under a fluorescence microscope.

Western Blot Analysis

HTM and SC cell lysates were prepared using 20 mM Tris buffer, pH 7.4, containing 1 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 M NaCl, 50 mM NaF, aprotinin (25 μg/ml), and leupeptin (25 μg/ml), and protein concentration was estimated by the Bradford method.[27] Equal amounts of protein (50 μg protein/lane) were separated by SDS-PAGE (10% or 12.5% acrylamide), followed by electrophoretic transfer of resolved proteins to nitrocellulose filters. Filters were then probed using antibodies specifically directed against Rho GTPase, Rho kinase, cofilin, myosin light chain, and LIM kinase, followed by incubation with peroxidase-linked secondary antibodies. Detection of immunoreactivity was carried out by enhanced chemiluminescence (ECL) according to manufacturer's recommendations (Amersham Biotechnology).

Myosin Light-Chain Phosphorylation

Myosin light-chain phosphorylation status in HTM and SC cells was determined by following the procedure described by Garcia et al.[28] Briefly, confluent cultures of control and drug-treated cells were extracted with 10% cold trichloroacedic acid, and precipitates obtained after centrifugation at 10,000 rpm were dissolved in 8 M urea buffer containing 20 mM Tris, 23 mM glycine, 10 mM dithiothreitol (DTT), saturated sucrose, and 0.004% bromophenol, using a sonicator. The urea-solubilized samples were separated on slab gels containing 10% acrylamide, 0.5% bisacrylamide, 40% glycerol, and 20 mM Tris, and 23 mM glycine. The running buffer for these gels was comprised of 20 mM Tris, 23 mM glycine, 2 mM DTT, and 2.3 mM thioglycolate in the upper chamber, whereas the lower chamber contained the same buffer minus DTT and thioglycolate. Proteins from these glycerol gels were transferred on to nitrocellulose filters in 10 mM sodium phosphate buffer, pH 7.6, using a Bio-Rad transfer apparatus. Nitrocellulose membranes were subjected to Western blot analysis using a rabbit polyclonal anti-myosin light-chain antibody. Blots were developed using peroxidase-conjugated goat anti-rabbit IgG and an ECL detection system.

SC Cell Monolayer Barrier Function

HRP diffusion through SC cell monolayers was evaluated using transwell cell culture chambers (collagen-coated polycarbonate filters with a pore size of 3 μM). SC cells seeded in the upper chambers of transwells were maintained in culture for a period of 10 days to obtain monolayers, with culture media being changed on alternate days. To measure monolayer barrier function, cell culture medium was replaced with media containing HRP (0.126 μM), before initiating drug treatment for 1 hour at 37°C. Media from the lower chamber were collected to monitor HRP enzyme activity by a colorimetric assay, as described by Lampugnani et al.[29] Results were expressed as percent change in enzyme activity over untreated controls, and statistical significance was evaluated by a paired *t*-test.

Aqueous Humor Outflow Facility

Porcine eyes (obtained freshly from a local abattoir) were perfused with Y-27632 by the standard constant pressure technique using a Grant stainless steel corneal fitting.[9] Initial baseline outflow measurements were established at 15 mm Hg and 25°C with perfusion medium containing Dulbecco's PBS (DPBS), pH 7.4, and 5.5 mM D-glucose. After this, the anterior chambers of test eyes were perfused with DPBS

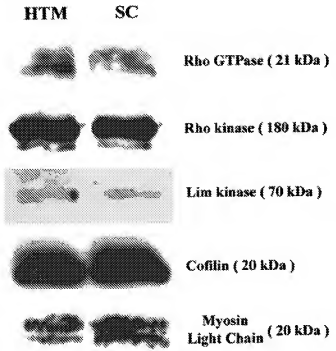

FIGURE 1. Immunochemical detection of Rho and its effector proteins in cell lysates of HTM and SC cells. An equal amount of total protein (50 μg/lane) was loaded from the HTM and SC lysates. All the proteins tested were found to be easily detectable in HTM and SC cells.

containing drug, whereas the contralateral fellow eye were perfused with DPBS alone, for a period of 5 hours. Outflow measurements were recorded at hourly intervals. Drug effects are expressed as the percentage change in outflow facility (compared with baseline values) over 5 hours, in drug-treated versus untreated paired controls (contralateral eyes). Values are expressed as mean ± SE. Data were analyzed by a paired two-tailed Student's *t*-test to determine significance. Samples that exhibited <0.2 μl/min, per mm Hg or >1.7 μl/min facilities were excluded from the study.

HTM and SC Morphology

At the end of a 5-hour perfusion period, sham control and drug-treated fellow eyes were fixed for histologic examination, by perfusing them with 2.5% glutaraldehyde and 2% formaldehyde at 15 mm Hg pressure. Tissue quadrants obtained from drug-treated and control eyes were fixed in 1.0% osmium tetra oxide in 0.1 M sodium cacodylate buffer and then stained with 1% uranyl acetate. Finally, sections obtained by microtomy (70 nm) were stained sequentially with $KMnO_4$ and Sato's stain and photographed using an electron microscope (Jeol Jem-1200 EX).

RESULTS

Expression of Rho Kinase and Its Accessory Proteins in HTM and SC Cells

The expression profiles of Rho kinase and its effector proteins were characterized by Western blot analysis of cell lysates derived from HTM and human SC primary cell cultures maintained in tissue culture. Antibodies specific for RhoA, Rho kinase, LIM kinase, cofilin, and myosin light chain were used in these studies. HTM and SC cells contained easily detectable amounts of RhoA, Rho kinase, LIM kinase, myosin light chain, and cofilin (Fig. 1).

Effect of Y-27632 on HTM and SC Cell Morphology and Cytoskeletal Organization

Treatment of HTM and SC cells (grown to confluence on gelatin-coated glass cover slips) with Rho kinase inhibitor (10 μM) for 1 hour led to cell- cell separation (retraction) and rounding up of cells (Fig. 2) in either the presence or absence of serum. These alterations were found to be reversible, with normal cell morphology being restored within 24 hours of drug withdrawal (data not shown). HTM cells appeared to exhibit more pronounced changes in cell shape in response to treatment with Y-27632. Treatment with Y-27632 (1, 4, or 10 μM) resulted in time- and dose-dependent changes in the relative staining of F-actin (phalloidin), focal adhesions (paxillin), and protein phosphotyrosine, in HTM and SC cells (data with 1 and 4 μM drug not shown). Figures 3A and 3B illustrate the

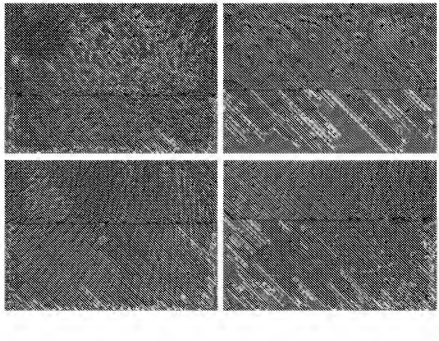

FIGURE 2. Rho kinase inhibitor induces morphologic changes in HTM and SC cells. Treatment with 10 μM Y-27632 treatment for 1 hour under serum-free conditions induced cell separation. Cells possessed a refractile appearance, with both HTM and SC cells exhibiting long filamentous cell bodies. These morphologic alterations were reversible within 24 hours of drug withdrawal. Magnification, ×100.

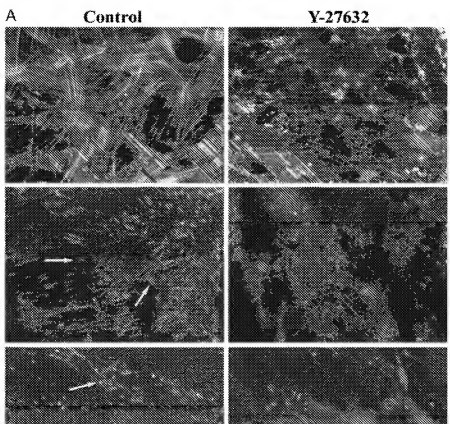

FIGURE 3. Y-27632 induces changes in actin stress fibers, focal adhesions (FAs), and protein phosphotyrosine (PY) profiles in HTM (A) and SC (B) cells. Cells were treated with 10 μM Y27632 for 1 hour at 37°C under serum-free conditions and subsequently stained for actin (with rhodamine-conjugated phalloidin), focal adhesions (with anti-paxillin antibody), or phosphotyrosine (with anti-phosphotyrosine antibody). In both HTM and SC cells, Y-27632 caused significant decreases in staining for actin stress fibers, focal adhesions, and protein phosphotyrosine, compared with untreated controls. Magnification, ×400. *Arrows*, focal adhesions and phosphotyrosin staining.

changes in F-actin, paxillin, and phosphotyrosine staining in HTM and SC cells treated with 10 μM Y-27632 under serum-free conditions. Similar effects were noted when HTM and SC cells were exposed to Y-27632 in the presence of serum (data not shown). To assess reversibility of these drug-induced cytoskeletal alterations, both HTM and SC cells were initially treated with drug (10 μM) for 90 minutes, after which serum-free culture medium containing drug was replaced with complete medium lacking drug. Cells were subsequently stained for F-actin, paxillin, and phosphotyrosine. Staining for F-actin, focal adhesions, and phosphotyrosine revealed that 24 hours after drug withdrawal there were no differences between untreated control cells and cells previously exposed to Y-27632, indicating complete reversibility of drug-induced cytoskeletal alterations (data not shown). Cells, both HTM and SC treated with 10 μM Y-26732 for 1 hour, showed no obvious cytotoxicity tested by fluorescein diacetate and propidium idodide staining (data not shown).

Effect of Y27632 on Myosin Light-Chain Phosphorylation in HTM and SC Cells

Myosin light-chain phosphorylation status is an important determinant of cellular contraction and relaxation and is regulated through the Rho/Rho kinase pathway.[16,21,24] To investigate the effects of Y-27632 on myosin light-chain phosphorylation status in HTM and SC cells, total protein precipitates obtained from drug-treated (lanes 2 and 4) and control samples (lanes 1 and 3) were subjected to urea-glycerol gel electrophoresis followed by Western blot analysis with polyclonal antibody to myosin light chain. Figure 4 shows the Y-27632–induced changes in myosin light-chain phosphorylation in HTM and SC cells. Lysates from both control HTM and SC cells (lanes 1 and 3) exhibited three immunoreactive bands representing the unphosphorylated (top), the mono-phospho- (middle), and di-phosphorylated (bottom) forms of myosin light chain. In control lysates, most of the myosin light chain existed either as the mono-phospho or di-phospho form, with a small proportion in the unphosphorylated form. Lysates obtained from drug-treated cells (lanes 2 and 4), in contrast, displayed only two bands, corresponding to the major unphosphorylated (top) and mono-phospho (middle) form, with a complete loss of the di-phospho myosin light chain (bottom). These data suggest that treatment of HTM and SC cells with the Rho kinase inhibitor triggers the dephosphorylation of myosin light chain.

Y27632 Modulates Monolayer Permeability/Barrier Function in SC Cells

Human SC primary cell cultures form confluent monolayers with strong cell–cell adhesions compared with HTM cells maintained in culture (see Fig. 2) and have been reported to maintain better barrier function, based on transendothelial electrical resistance measurements.[30] Furthermore, because HTM cells found on the beams of the TM in vivo do not exist as monolayers, we conducted these assays with SC cells alone. SC cell monolayers ($n = 7$ transwells) treated with Y-27632 (10 μM) in serum containing media at 37°C displayed a significant increase (80%; $P < 0.005$) in HRP permeability over untreated

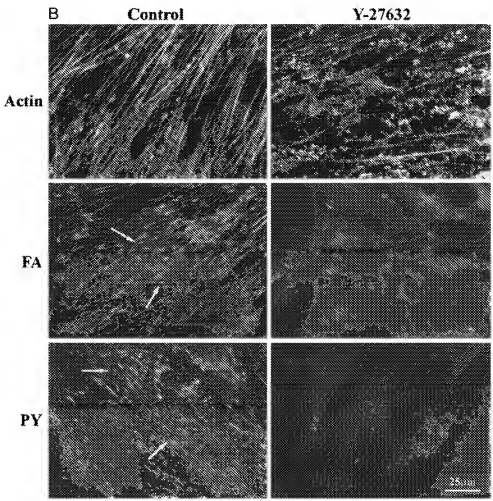

FIGURE 3. (continued)

controls (Fig. 5). Phalloidin staining assays revealed that this change in barrier function was associated with decreased actin stress fibers and cell–cell detachment in SC cells growing in transwells (data not shown).

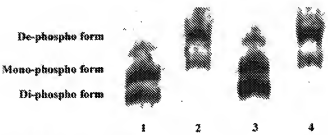

FIGURE 4. Y-27632 decreases myosin light-chain phosphorylation in HTM and SC cells. TCA-precipitated proteins from control and drug-treated (10 μM for 1 hour) HTM and SC cells were separated on urea-glycerol gels, followed by electrophoretic transfer to nitrocellulose filters. Western blot analyses were developed using anti-myosin light-chain antibody. Control samples (*lanes 1* and *3*: HTM and SC, respectively) contain three specific immunopositive bands of myosin light chain, corresponding to the unphosphorylated, monophosphorylated, and diphosphorylated forms of the protein. In contrast, samples obtained from drug-treated cells (*lanes 2* and *4*: HTM and SC, respectively) contain only two myosin light-chain bands, representing the unphosphorylated and mono-phosphorylated forms, with complete disappearance of diphospho form, indicative of the inhibition of myosin light-chain phosphorylation by Y-27632.

Effect of Y-27632 on Aqueous Outflow Facility of Enucleated Porcine Eye

Cadaver porcine enucleated eyes obtained from a local abattoir were perfused with Y-27632 (10, 50, or 100 μM) at a constant pressure of 15 mm Hg, after establishing the baseline outflow facility with PBS buffer containing glucose at 25°C. Basal rates

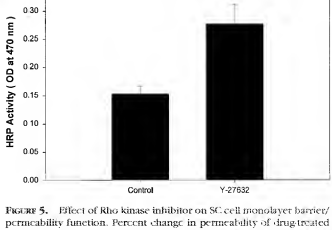

FIGURE 5. Effect of Rho kinase inhibitor on SC cell monolayer barrier/permeability function. Percent change in permeability of drug-treated cell monolayers over the corresponding untreated controls was 80% ($P < 0.005$). Values are mean of seven samples; error bars, SE.

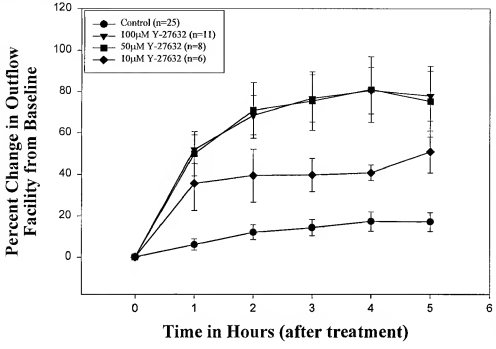

FIGURE 6. Y-27632 increases outflow facility in enucleated porcine eyes. Contralateral paired eyes were perfused with PBS containing 5.5 mM glucose under a constant pressure of 15 mm Hg at 25°C. After establishing the initial base line outflow facility, eyes were perfused either with Rho kinase inhibitor or perfusion medium alone for 5 hours, with outflow facility being monitored at hourly intervals. Changes in outflow facility of drug-treated eyes are expressed as percent change over the initial base line outflow facility values. Symbols represent mean values, error bars, ± SE. Differences in outflow facility between control and drug perfused eyes (10, 50, and 100 μM) were found to be statistically significant, with $P <$ 0.04, 0.002, and 0.0002, respectively.

of outflow facility (μl/min per mm Hg) in the control and Y-27632-perfused groups (10, 50, and 100 μM) were 1.09 ± 0.007 (n = 25), 0.84 ± 0.16 (n = 6), 0.80 ± 0.15 (n = 8), and 0.96 ± 0.10 (n = 11), respectively (mean ± SE). Outflow facility was observed to increase significantly (10 μM, $P <$ 0.04; 50 μM, $P < 0.002$; and 100 μM. $P < 0.0002$) after 1 hour of Y-27632 treatment (30%–50% over the baseline outflow facility). Outflow facility continued to increase (80% increase after 3 hours of perfusion with 50 or 100 μM Y-27632), reaching a plateau thereafter (Fig. 6), whereas a 40% increase was observed after 3 hours of perfusion with 10 μM drug. Fellow paired control eyes perfused with PBS buffer alone showed only a marginal increase (typical washout effect of 10%–18%) in outflow facility over the corresponding initial baseline outflow facility values. Thus, there appears to be a good dose-dependent increase in outflow facility between 10 and 50 μM Y-27632.

Y27632-induced Structural Changes in Tissues of the Outflow Pathway

No cell loss and accumulation of cell debris were observed in the TM of eyes perfused with either 50 or 100 μM drug for 5 hours, and the integrity of the inner wall of aqueous plexi was observed to be intact, as assessed by transmission electron microscopy, indicating no cytotoxic effects of Y-27632 (Fig. 7). However, compared with control specimens that exhibited compact trabecular beams and juxtacanalicular area, drug-treated samples demonstrated widening of the extracellular spaces, particularly the optically empty areas in the juxtacanalicular tissue (indicated by the arrows in Fig. 7). Additionally the entire TM appeared distended. Interestingly, "more giant vacuoles" (inner wall invagination) were observed in the inner wall of Schlemm's canal in drug-perfused specimens compared with controls.

Discussion

In this study we sought to evaluate the potential role of the Rho/Rho kinase signaling pathway in modulation of aqueous humor outflow facility, and data presented here demonstrate that inactivation of Rho kinase by Y-27632 increases aqueous outflow facility in perfused porcine eyes and that this effect correlates with increased paracellular permeability measured in SC cell monolayers as well as cellular relaxation and cytoskeletal reorganization of cells of the outflow pathway. Changes in cell morphology, intercellular junctions, focal adhesions, and contractile/relaxation characteristics of HTM, juxtacanalicular, and SC cells are often associated with changes in aqueous humor outflow facility.[4–14] Each of these cellular changes can be influenced directly or indirectly by actomyosin cytoskeletal organization.[4–13] Furthermore, studies using myosin light-chain kinase inhibitors such as H-7, M-9, and BDM suggest that myosin light-chain phosphorylation may play a critical role in the cytoskeleton-based regulation of HTM and SC cellular contraction/relaxation and aqueous outflow through the TM and Schlemm's canal.[4,7,9]

Because myosin light-chain phosphorylation is critical for cellular contraction[31–35] and for the formation of actin stress fibers and focal adhesions,[16,18] we chose to study the effects of Y-27632, a specific inhibitor of Rho kinase[21,25] on cell morphology, actin stress fibers and focal adhesions, phosphotyrosine status, and myosin light-chain phosphorylation in HTM and SC cells and to correlate these effects with changes in monolayer permeability of SC cells and outflow facility in enucleated porcine eyes.

Treatment of HTM and SC cells with Y-27632 induced changes in cell morphology and led to decreases in actin stress fibers and focal adhesions and in levels of protein phosphotyrosine, compared with untreated controls (Figs. 2 and 3). Myosin light-chain phosphorylation was also found to be dramatically lowered in Y-27632–treated cells (Fig. 4). These cytoskeletal changes and the decrease in myosin light-chain phosphorylation were associated with increased SC cell monolayer permeability and increased outflow facility in porcine eyes (Figs. 5 and 6). SC cell monolayers treated with Rho kinase inhibitor revealed extensive morphologic changes such as cell–cell detachment and actin depolymerization, changes that are associated with increased permeability of HRP through SC cell monolayers. However, cell–cell detachment was not evident in the inner wall of aqueous plexi in drug-perfused por-

cine eyes, which revealed a significant increase in outflow facility. (Fig. 7). Y-27632–induced changes were not attributable to drug-induced cytotoxicity, because these cellular effects were completely reversed upon drug withdrawal, as evidenced by a lack of obvious cytotoxic effects in cell culture.

Activation of Rho GTPase by agonists (thrombin, lysophosphatidic acid [LPA], endothelin, and growth factors) has been found to stimulate formation of actin stress fibers, integrin-mediated cell–substratum adhesions (focal adhesions), protein tyrosine phosphorylation, myosin light-chain phosphorylation, and contraction of smooth muscle.[15,33,34,35] Earlier studies from our laboratory have documented that perfusion of porcine eyes with thrombin and LPA produces a decrease in measured outflow facility, an effect that correlated with decreased SC cell monolayer permeability, increased formation of actin stress fibers and focal adhesions, and myosin light-chain phosphorylation (Kumar J, Rao PV, Epstein DL, unpublished data).

Thus, we have noticed a striking correlation between activation of Rho GTPase and decreased outflow facility and also have established that inhibition of Rho kinase leads to increased outflow facility. On the basis of these contrasting effects, we propose the following mechanistic rationale for the involvement of Rho/Rho kinase in modulation of aqueous humor outflow facility through induced changes in cellular "tone" (contraction/relaxation).

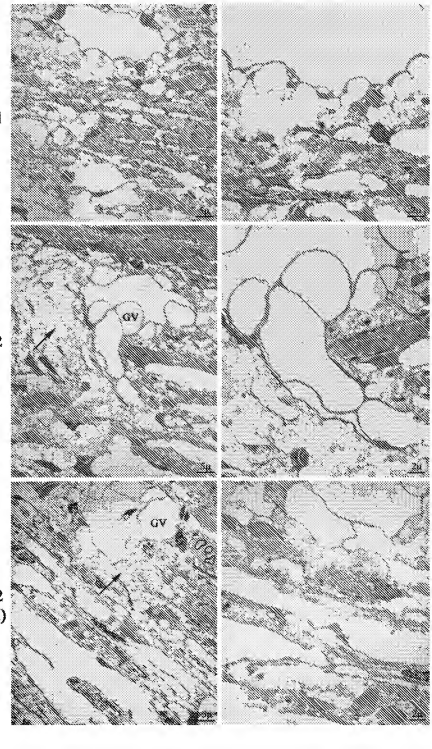

FIGURE 7. Effects of Y-27632 perfusion on the integrity of trabecular meshwork of enucleated porcine eyes. Enucleated porcine eyes perfused with Y-27632 (50 or 100 μM) for 5 hours at 25°C were fixed for histologic examination by electron microscopy. Although the lining of the inner wall of aqueous plexi showed no obvious breaks, there appeared to be more empty spaces in the juxtacanalicular tissue and more giant vacuoles (GV) compared with controls in the inner wall of aqueous plexies, in drug-perfused specimens. Furthermore, the trabecular beams in drug-treated specimens appeared less compact compared with those from control samples, suggesting some distention of outflow pathway. *Left* and *right*: with low (×1000) and high (×2500) magnification of the same specimen, respectively. *Arrows*, the optically empty spaces in JCT.

Decreased myosin light-chain phosphorylation resulting from inhibition of Rho kinase leads to lowered actomyosin-based cellular contraction and to cell–cell detachment and loss of actin stress fibers and focal adhesions as well as a decrease in protein phosphotyrosine levels in HTM and SC cells (Fig. 3). These changes in the actomyosin cytoskeleton and cell adhesion properties result in relaxation of HTM and SC cells and in tissues of the outflow pathway overall. This change in tone and cell–cell separation could potentially lead to increased permeability of SC cells (via a paracellular pathway or transcellular pores). Cellular relaxation may also result in enhanced flow through the JCT outflow channels because of decreased cell–cell adhesion and cell–ECM interactions. In contrast, it appears plausible that Rho GTPase-activated cellular contraction leads to an increase in outflow resistance by related opposite influences on elements of the outflow pathway. The fact that cellular relaxation in SC cells exhibits a functionally relevant association with enhanced SC monolayer permeability lends support to our hypothesis regarding the role of Rho GTPase-mediated cellular contraction in decreasing outflow facility[13,14] (Kumar J, Rao PV, Epstein DL, unpublished data). Although SC and HTM cells are thought to be endothelial in nature, their responses to LPA and thrombin are distinct from that of human endothelial cells.[34–36] Human endothelial cells have been shown to retract and contract in response to thrombin treatment, with a resultant increase in paracellular diffusion through cell monolayers.[34,36] In contrast, SC cells do not exhibit any change in cell morphology upon LPA or thrombin stimulation but do exhibit decreases in permeability, suggestive of cell-type–specific differences in contractile properties between SC and endothelial cells. Finally, it is also possible that Y-27632 potentially affects tight junctional protein assembly[37,38] and thereby directly influence paracellular flow mechanisms (through preferential flow channels) in the inner wall of the Schlemm's canal.[39]

In support of our contention, endothelin, a physiological agonist of Rho GTPase-mediated signaling,[15,16] has been shown to decrease outflow facility in perfusion studies.[14] Increased levels of endothelin have also been reported in aqueous humor samples of glaucoma patients.[40] Additionally, a human mutation in myotonic dystrophy kinase is associated with lowered ocular pressure.[41,42] The catalytic domain of myotonic dystrophy kinase shares a 72% sequence homology with the kinase domain of Rho kinase.[43] Given the diversity of morphologic and cytoskeletal events regulated by Rho kinase, it is reasonable to infer that Rho kinase–mediated signaling pathways may play an important role in the physiological regulation of ocular pressure.

Another potential consideration is that, structural/physical changes induced in the outflow pathway including both the JCT and SC inner wall by these mechanisms might also influence the direction of the flow pathway and/or the available free space for fluid to seep through and thereby directly affect the filtering property/capacity of the whole outflow pathway tissue. As shown in Figure 7, perfusion of Y-27632 causes some apparent distention of the outflow pathway and an increase in optically empty space in the JCT, including apparently enhanced formation of giant vacuoles[2] in the inner wall. These structural changes could potentially influence the direction or dimensions of the outflow pathway directly, similar to observed influences of H-7,[7,13] iodoacetamide,[44] pilocarpine,[3] and EDTA.[45]

Finally, this study underscores the importance of Rho/Rho kinase–mediated signaling pathways in the regulation of actomyosin cytoskeletal dynamics of HTM and SC cells and in maintenance of aqueous humor outflow facility. Thus, physiological and pharmacological agents that influence Rho/Rho kinase-mediated signaling pathways represent a potential therapeutic means to treat elevated ocular pressure in glaucoma patients.

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Vasanth Rao

To: David L Epstein/EyeCenter/mc/Duke@mc
Subject: Re: rho kinas inhibitor* ***

Statins effect cell shape, depolymerize actin stress fibers and decrease myosin light chain phosphorylation in different cell type including TM cells by impairing Rho/Rho kinase activities. This effect of statins is independent of cholesterol and is through isoprenylation modification. Therefore, statins including (Zocor (Merck) , Parvacore (Bristal Maiyer), Mevacore(Merck) and Lipatore(Pfizer) should be able to lower IOP but we have not screened these individual drugs. Most of my work was with lovastatin (Merck) and it was in vitro. We have not perfused these compounds to test their effect on outflow facility. One of the draw backs for this is, these compounds take minimum of 18 hours to exert their effect on cell shape and cytoskeletal organization. Therefore, we need to use the organ culture perfusion system not the Grant system.

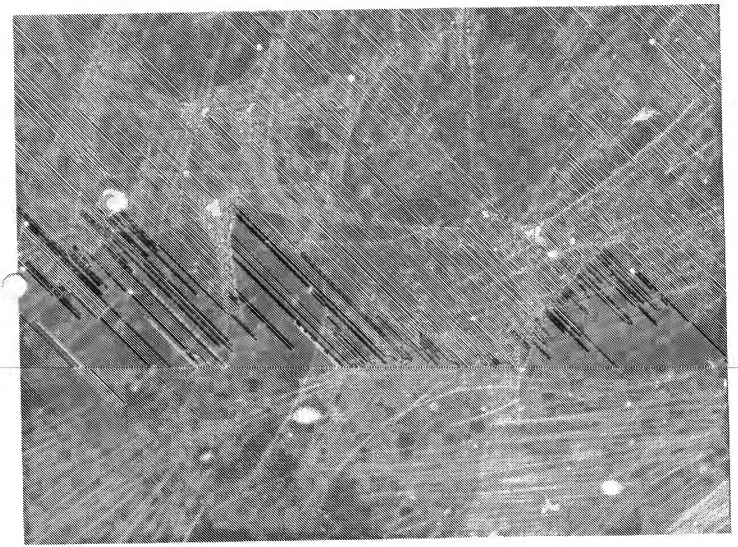
My RPB grant was based on these ideas. But resources, personnel and space are the constrains for this type of screening work. I think there is one existing patent on the statins for eye diseases but not related to cytoskeleton or Rho GTPases. Thanks. Vasanth

EXHIBIT B

EXHIBIT C

Time Course effect

control



24 hrs

South Louisiana